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FACSIMILE

Date: January 23, 2004 To: Examiner Kumar USPTO Facsimile No.: 703-746-3125 Telephone No..	From: Richard J. Traverso **MILLEN, WHITE, ZELANO & BRANIGAN, P.C.** **Arlington Courthouse Plaza I** **2200 Clarendon Blvd., Suite 1400** **Arlington, VA 22201 (U.S.A.)** **(Fax: 703-243-6410)** Writer's Direct Dial: 703 - 812-6310 Writer's Internet Address: traverso@mwzb.com
Re: Bayer-0006-P01 USSN: 09/776,936	

Total No. of Pages: 18; if you do not receive all pages, please call (703) 243-6333

Dear Examiner Kumar:

Attached is the complete copy of the Bruder reference we discussed by phone and a copy of the Russian Examination Report which cites U.S. patent 3,284,433, EP 709225A1 and WO 96/25157. I have also included an English translation of the Russian Examination Report. If you require further information, please let us know.

Respectfully submitted,

Richard J. Traverso, Reg. No. 30,595

C_{1-10}alkoxy, halogen, OH, $-SCH_3$, NO_2,

or a pharmaceutically acceptable salt thereof,

with the proviso that if X is -O- or -S-, $R^{3'}$ and $R^{6'}$ are H, and Y is phenyl unsubstituted by OH, then R^6 is alkoxy.

Therefore, the Applicant is invited to restrict the claims to the above limitations or provide additional data in support of the claims.

There is no representative of the inventive groups of compounds characterized in terms of pKa. At the same time, as can be seen from the application documents and the most relevant prior art reference that describes structurally close compounds, the value of pKa is a feature essential for the activity claimed (see also WO96/25157, published 22.08.1996, 117 pages). Therefore, the Applicant is invited to submit the above data.

The Applicant is further invited to revise the claims in order to remove known compounds from the scope of the claims. In particular, the claims as filed encompass a whole series of known compounds. For example, if R^3-R^6 and $R^{3'}$-$R^{6'}$ are simultaneously hydrogen, or one or more of them is/are an alkyl or halogen, nitro group or X-Y wherein X is as defined above and Y is an optionally substituted phenyl, then the structural formula would cover some known compounds: unsubstituted diphenyl ureas or alkyl, alkoxy, nitro or halogen substituted diphenyl urea derivatives. For example, U.S. Patent 3,284,433, published 08.11.1996 (5 pages), teaches obtaining 4-phenoxycarbanylides wherein the phenyl rings may be substituted by halogen atoms and a nitro group. Claim 4 of the aforesaid patent document describes the compound as defined in claim 12 of the present invention. The reference WO96/25157 previously mentioned, too, describes various diphenyl substituted ureas with the phenyl rings comprising the same radicals as employed by the present invention. EP 709225 A1 published 01.05.1996 (64 pages) discloses obtaining compounds of formula V on page 4, compound D1 on page 13 and other compounds on pages 14, 15, which are covered by the general structural formula of the inventive compounds as well.

Regarding dependent claims 2-11, the Applicant is invited to adapt them to the amendments to be made to claim 1 (see the requirements of paragraph 19.4(7) of the aforesaid Rules concerning the structure of claim and manner of claiming and the requirements of paragraph 3.3.2.5(3) specifically concerning dependent claims).

Claim 12 is directed to a single compound which is disclosed in U.S. Patent 3,284,433, as mentioned above. That is, claim 12 does not meet the novelty criterion.

Regarding claim 13, directed to a pharmaceutical composition, the Applicant is invited to designate the mode of activity in this claim, keeping within the original disclosure of the invention. Subject to revision of the groups of compounds according to claim 1 and presentation of activity and toxicity data as well as examples of the composition (see the requirements of paragraphs 19.5.1(2) and 3.2.4.5(3) of the aforesaid Rules), the Applicant is invited to consider restating claim 13 to read:

13. A pharmaceutical composition having properties of inhibitor of a cancerous cell growth mediated by raf kinase, comprising a compound of claim 1, and a physiologically acceptable carrier.

Claim 14 is directed to a pharmaceutical composition comprising a compound of claim 12. However, as mentioned above, claim 12 is anticipated by the prior art and thus has to be canceled. In that case, claim 14 would no longer meet the requirement of unity of invention for the lack of a single inventive concept between claim 14 and claim 1 (see paragraphs 19.4(5) and 2.3 of the aforesaid Rules concerning the unity of invention requirements). Therefore, claim 14 has to be canceled.

The subject matter of claims 15-19, directed to a method of inhibiting a cancerous cell growth mediated by raf kinase, is disclosed too generally. There is a description of testing procedures, but no indication of particular compounds tested, their activity and toxicity data. Furthermore, these claims cover the group of compounds of formula I and compounds of claim 12, as well as compounds not included in claim 1 or claim 12. At the same time, the Applicant fails to give any information concerning the compounds not included in claim 1 or claim 12. That is, there is no information provided as to the method of obtaining, physico-chemical characteristics and activity data, or indication of references in which such compounds or method of obtaining the same would be described. The Examining Authority suggests that claim 15 be limited to the compounds of formula I according to claim 1, taking into account the application documents and the foregoing remarks.

Dependent claims 16-19 should be adapted to independent claim 15 (see the requirements of paragraph 19.4(7) and 3.3.2.5(3) concerning the structure of claims and the manner of claiming).

3. In view of the foregoing, the Applicant is invited to analyze the statements of the Examining Authority, present additional data, amended claims and amended description incorporating all the amendments and additions to be made (see paragraphs 19.4(7), 19.6(5) of the Rules).

Department for organic compounds technology
Leading state patent examiner S.L. Polyakova

РОССИЙСКОЕ АГЕНТСТВО
ПО ПАТЕНТАМ И ТОВАРНЫМ ЗНАКАМ
(РОСПАТЕНТ)

ФЕДЕРАЛЬНЫЙ ИНСТИТУТ
ПРОМЫШЛЕННОЙ СОБСТВЕННОСТИ

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Телефон 240 60 15. Телекс 114818 ПДЧ. Факс 243 33 37

Форма № 10 ИЗ, ПО-2000

На № 200429 от

(21) Наш № 2000120165/04(021682)

При переписке просим ссылаться на номер заявки и сообщить дату получения данной

ЗАПРОС

(21) по заявке № 2000120165/04(021682)

(22) Дата поступления заявки 22.12.1998

(86) Заявка № PCT/ US98/26081 от 22.12.1998 (96) Заявка №EA

(71) Заявитель(и) БАЙЕР КОПЭРЕЙШН, US

(51) МПК C07C275/24, C07D 213/02,333/02,A61K31/17,31/38,31/44 (51) МКПО

Для обеспечения возможности дальнейшего рассмотрения заявки экспертиза предлагает заявителю представить материалы, документы, сведения в связи с поставленными вопросами, мнение относительно приведенных в запросе доводов, замечаний, предложений.

Ответ на запрос должен быть представлен в установленный пунктом 8 статьи 21 Патентного закона Российской Федерации (введен в действие 14.10.92) срок. По просьбе заявителя, поступившей до истечения этого срока, он может быть продлен при условии представления документа об уплате пошлины в установленном порядке.

В случае непоступления в указанный срок ответа на запрос или при непродлении этого срока заявка будет признана отозванной.

ВОПРОСЫ, ДОВОДЫ, ЗАМЕЧАНИЯ, ПРЕДЛОЖЕНИЯ

(См. на оборотe)

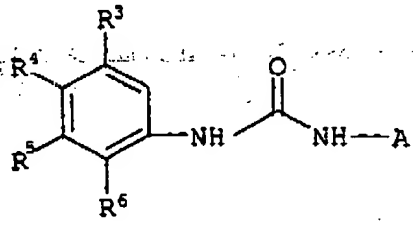
05		041501

1.Предложено соединение формулы I (см. п.п.1-11 формулы изобретения), соединение формулы, указанной в п.12, фармацевтическая композиция (варианты)(см. п.п.13,14) и способ подавления роста опухолевых клеток (см.п. п.15-19).

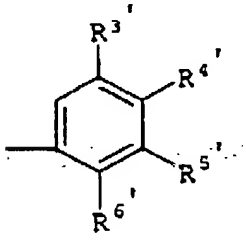
Заявитель просит установить конвенционный приоритет 22.12.1997, на основании заявки № 08/996344, поданной в патентное ведомство США.

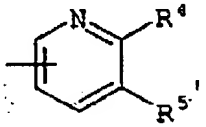
2.Согласно требованию п.19.5.1(2) Правил-1 при установлении возможности использования изобретения, проверяется описаны ли в материалах заявки средства и методы с помощью которых возможно осуществления изобретения в том виде, как оно охарактеризовано в любом из пунктов формулы изобретения, и действительно ли возможна реализация указанного заявителем назначения. Более подробно указанные требования изложены в пункте 3.2.4.5(3) и (2) Правил-1, если изобретение относится к новым химическим соединениям , фармацевтической композиции и способу лечения.

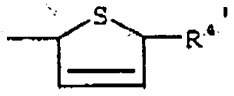
В материалах заявки отсутствуют показатели активности представителей групп соединений с разными по химической природе радикалами,а также отсутствуют данные по токсичности предлагаемых соединений. Заявителю следует представить такие данные. Имеющиеся материалы заявки раскрывают способ получения, физико-химические характеристики групп соединений формулы I



где A означает







или

Для сведения заявителя

1.При запросе копий противопоставленных источников информации необходимо представить документ об оплате услуги за предоставление испрашиваемого количества страниц, указанных в тексте по действующим тарифам:-согласно договору

непатентная литература за 1 страницу

- патентная литература за 1 страницу

2.Правила составления, подачи и рассмотрения заявки на выдачу патента на изобретение введены в действие 16.10.98 (Правила - 1)

3.Правила составления, подачи и рассмотрения заявки на выдачу патента на промышленный образец введены в действие 23.10.98(Правила -2)

R^3, R^4, R^5 и R^6 каждый независимо означает H, галоген, NO_2, C_{1-4}алкил, по выбору замещенный галогеном вплоть до полного замещения; C_{1-4}алкокси, по выбору замещенный галогеном вплоть до полного замещения; C_5гетероарил,содержащий в качестве гетероатома азот или серу и по выбору замещенный C_{1-4}алкилгруппами, и один из R^3-R^6 по выбору означает -X-Y;

или два соседних R^3-R^6 могут быть объединены с образованием фенильного или гетероарильного -$S(O_2)$-содержащего цикла с 5 атомами в цикле,

$R^{4'}$, $R^{5'}$ и $R^{6'}$ независимо означают H, галоген, C_1-C_4алкил, по выбору замещенный галогеном вплоть до полного замещения,C_1-C_4алкокси, по выбору замещенный галогеном вплоть до полного замещения или -X-Y, и любой из R^4, R^5 или R^6 означают -X-Y, или 2 соседних заместителя из $R^{4'}$, $R^{5'}$ $R^{6'}$ объединены с образованием гетероарильного серусодержащего кольца, содержащего 5 атомов, по выбору замещенного следующими группами: C_{1-4}алкил, C_{1-4}алкокси,

$R^{6'}$, кроме того, означает NO_2;

R^1 означает C_{1-10}алкил, по выбору замещенный галогеном вплоть до полного замещения;

$R^{3'}$ означает H, галоген, C_1-C_{10}алкил, по выбору замещенный галогеном вплоть до полного замещения, C_1-C_{10}алкокси, по выбору замещенный галогеном вплоть до полного замещения;

X означает -CH_2-, -S-, -$N(CH_3)$-, -NHC(O)-, -CH_2-S-, -S-CH_2, -C(0)-, или -O-; и X, кроме того, означает простую связь, где Y означает пиридил; и

Y означает фенил, пиридил, нафтил, пиридон, пиразин, пиримидин, бензодиоксан, бензопиридин или бензотиазол, каждый по выбору замещен следующими группами: C_{1-10}алкил, C_{1-10}алкокси, галоген, OH, -SCH_3, NO_2,

или их фармацевтически приемлемая соль,

при условии, что, если X означает -O- или -S-, $R^{3'}$ и $R^{6'}$ означают H, а Y означает фенил, незамещенный OH, то R^6 означает алкокси.Поэтому экспертиза предлагает ограничить притязания имеющимися материалами по п.1 формулы или представить соответствующие дополнительные данные.

При этом ни для одного из представителей полученных групп соединений не указана величина pKa.В то же время, как видно из материалов заявки и из ближайшего аналога, описывающего близкие по структуре соединения, значение указанной величины pKa

является существенным признаком для указанного вида активности (см.также WO96/25157 опублик. 22.08.1996, всего 117 стр.)Поэтому экспертиза предлагает представить указанные данные.

При уточнении формулы изобретения экспертиза также предлагает заявителю исключить из объема притязаний известные соединения. В частности,в том виде, как представлена формула изобретения, она включает целый ряд известных соединений. Например, если R^3-R^6 и $R^{3'}$- $R^{6'}$ одновременно означают водород,или один или несколько из них означают алкил или галоген,нитрогруппу,или X-Y, где X имеет разные значения, указанные выше, а Y означает возможно замещенный фенил, то под предлагаемую структурную формулу подпадают известные соединения- незамещенные дифенилмочевины, или алкил, алкокси, нитро или галогензамещенные производные дифенилмочевины. Например,в патенте US 3284433, опублик. 08.11.1966г.(всего 5 стр.)описаны и получены 4-феноксикарбанилиды,в которых фенильные кольца могут быть замещены атомами галогена и нитрогруппой. В пункте 4 указанного патента описано соединение по п.12 формулы настоящего изобретения.В WO96/25157, указанном выше,также описаны различные дифенилзамещенные мочевины, в которых фенильные кольца, также, как и в предлагаемых соединениях, могут содержать идентичные значения радикалов.В EP 709225A1, опублик.01.05.1996(всего 64 стр.) описаны и получены соединения формулы V стр.4,стр. 13- соединение D1 и другие соединения на стр. 14,15), которые также входят в общую структурную формулу соединений настоящего изобретения.

Зависимые пункты формулы 2-11 экспертиза предлагает привести в соответствие с уточненным независимым пунктом 1(см. требования п.19.4(7) Правил-1 к структуре и изложению формулыизобретения и п.3.3.2.5(3) Правил-1, разъясняющий требования к зависимым пунктам формулы).

Пункт 12 формулы изобретения относится к одному соединению, которое, как указано выше, описано в патенте US 3284433. То есть п.12 формулы не соответствуетусловию патентоспособности-новизна.

В пункт 13 формулы изобретения , относящийся к фармацевтической композиции, экспертиза предлагает включить вид активности, с учетом имеющихся материалов заявки. После уточнения групп соединений по п.1 формулы, а также представления показателей активности, данных по токсичности, и примеров композиций (см. требования п.19.5.1(2) Правил-1 и п.3.2.4.5(3)-Правил-1), экспертиза предлагает изложить п. 13 в следующей редакции:

13.Фармацевтическая композиция,обладающая свойствами ингибитора роста опухолевых клеток, опосредствованного киназой RAF, содержащая соединение по п.1 и физиологически приемлемый носитель.

Пункт 14 формулы изобретения , относится к фармацевтической композиции, включающей соединение по п.12 формулы.Однако, как указано выше, соединение по п.12 формулы было ранее описано и должно быть исключено из притязаний заявителя.В этом случае, п.14 формулы будет нарушать единство изобретения, поскольку не связано с п.1 формулы единым изобретательским замыслом (см. п.19.4(5) Правил-1 и п.2.3 Правил-1, разъясняющий требования к единству изобретения). Поэтому п.14 должен быть исключен из формулы изобретения.

Пункты 15-19 формулы , относящиеся к способу подавления роста опухолевых клеток, опосредствованного киназой RAF раскрыт в общем виде. Указана методика испытаний ,но не указано какие соединения испытаны и их показатели активности, а также данные по токсичности.Кроме того,эти пункты , включают группу соединений формулы I и соединений по п.12, а также соединений не включенных в п.1 или п.12 формулы. При этом заявителем не приводится каких-либо данных относительно соединений не включенных в п.1 или п.12 формулы изобретения. Т.е не приводится данных о способе их получения, физико-химических характеристик, а также показателей активности, либо литературных данных, в которых описаны эти соединения и способ их получения. Экспертиза предлагает ограничить круг соединений, используемых в п.15 соединениями формулы I по п.1 формулы ,с учетом имеющихся материалов заявки и вышеизложенного.

Зависимые пункты формулы 16-19 следует привести в соответствие с независимым п.15 формулы (см. требования п.19.4 (7) к структуре и изложению формулы изобретения, а также п.3.3.2.5(3) Правил-1,разъясняющий эти требования).

3.На основании изложенного признано необходимым предложить заявителю проанализировать доводы экспертизы, представить дополнительные данные, уточненную формулу изобретения и откорректированное описание включающее все изменения и дополнения (см. п.19.4(7), 19.6(5) Правил-1).

Просим подтвердить возможность использования указанного в заявлении почтового адреса для переписки с заявителем, а в случае изменения его, сообщить об этом

Ведущий государственный патентный
эксперт отдела технологии
органических соединений С.Л.Полякова
240 25 27

Client of 7/29

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OF THE INDUSTRIAL PROPERTY**
Berezshkovskaya nab., 30, korp. 1, Moscow, G-59, GSP-5, 123995
Telephone 240-60-15 Telex 114818 PDCH Fax 243-33-37

Re: 200429

(21) Our № 2000120165/04(021682)

In correspondence please refer to the application number and indicate date of receipt of this communication

REQUEST

(21) Application № 2000120165/04(021682)

(22) Filing date: December 22, 1998

(86) Application PCT/US98/26081 of December 22, 1998

(71) Applicant(s): Bayer Corporation, US

(51) IPC: 7 C07C 275/24, C07D 213/02, 333/02, A61K 31/17, 31/38, 31/44

To provide the possibility of further consideration of the application, the Examiner invites the Applicant to submit materials, documents, data in connection with questions raised and voice his opinion of the arguments, remarks and proposals contained in the request

A reply to the request is to be given within a statutory period of time under the Patent law of the Russian Federation art. 21, § 8. At the Applicant's request received before the expiry of this period of time it can be extended provided a document is submitted on payment of fee in accordance with established procedure

In case if the reply to the request is not received by a specified date or this time period is not extended the application will be regarded as withdrawn.

QUESTIONS, ARGUMENTS, REMARKS, PROPOSALS

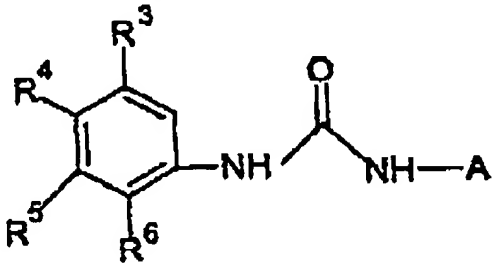
1. There are claimed a compound of formula I (claims 1-11), a compound of the formula defined in claim 12, a pharmaceutical composition (variants, see claims 13, 14) and a method for inhibiting a cancerous cell growth (claims 15-19).

The Applicant claims a convention priority of December 22, 1997 as the filing date from earlier application 08/996,344 filed with the U.S. Patent and Trademark Office.

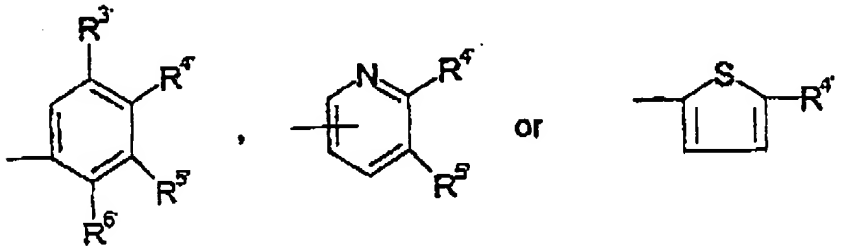
2. In accordance with paragraph 19.5.1(2) of the Rules on drafting, filing and examination of a patent application, it should be ascertained during the feasibility assessment whether the application documents describe any means and techniques allowing the invention as defined in each of the claims to be put into practice and whether the invention can actually achieve the purpose stated by the applicant. Paragraphs 3.2.4.5(3) and (2) of the aforesaid Rules specify the above

requirements with respect to novel chemical compounds, pharmaceutical compositions and methods of medical treatment.

The application fails to present any activity characteristics for representatives of the groups of compounds which have chemically different radicals, and toxicity data for the compounds claimed. The Applicant is invited to provide such data. The application documents disclose a method of obtaining groups of compounds of formula I and physico-chemical characteristics thereof



where A is



R^3, R^4, R^5 and R^6 are each, independently, H, halogen, NO_2, C_{1-4}alkyl optionally substituted by halogen up to perhaloalkyl; C_{1-4}alkoxy optionally substituted by halogen up to perhaloalkoxy; C_5-heteroaryl containing nitrogen or sulfur as heteroatom and optionally substituted by C_{1-4}alkyl groups, and one of R^3-R^6 is optionally -X-Y;

or two adjacent R^3-R^6 can together be a phenyl or heteroaryl **-S(O_2)-** containing ring with 5 atoms,

$R^{4'}$, $R^{5'}$ and $R^{6'}$ are each, independently, H, halogen, C_{1-4}alkyl optionally substituted by halogen up to perhaloalkyl, C_{1-4}alkoxy optionally substituted by halogen up to perhaloalkoxy, or -X-Y, and any of R^4, R^5 or R^6 is -X-Y, or two adjacent $R^{4'}$, $R^{5'}$ and $R^{6'}$ can together be a heteroaryl sulfur containing ring with 5 atoms, optionally substituted by C_{1-4}alkyl, C_{1-4}alkoxy,

$R^{6'}$ is additionally NO_2;

R^1 is C_{1-10}alkyl optionally substituted by halogen up to perhaloalkyl;

$R^{3'}$ is H, halogen, C_{1-10}alkyl optionally substituted by halogen up to perhaloalkyl, C_{1-10}alkoxy optionally substituted by halogen up to perhaloalkoxy;

X is $-CH_2-$, -S-, $-N(CH_3)-$, -NHC(O)-, $-CH_2-S-$, $-S-CH_2-$, -C(O)-, or -O-; and X is additionally a single bond where Y is pyridyl; and

Y is phenyl, pyridyl, naphthyl, pyridone, pyrazine, pyrimidine, benzodioxane, benzopyridine or benzothiazole, each optionally substituted by C_{1-10}alkyl.

requirements with respect to novel chemical compounds, pharmaceutical compositions and methods of medical treatment.

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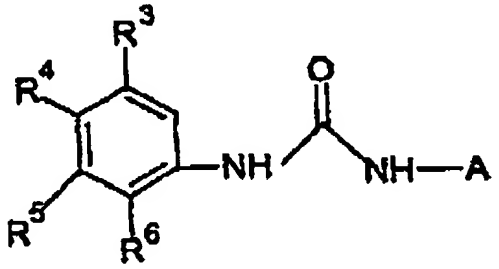

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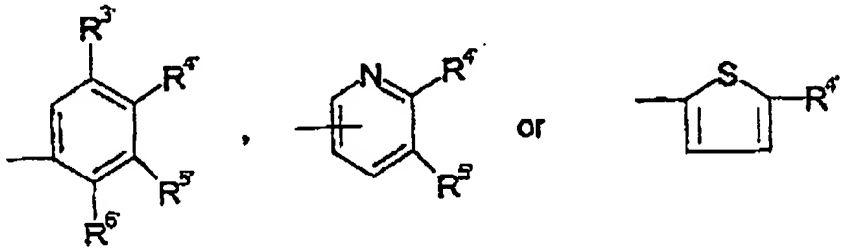

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X is $-CH_2-$, -S-, $-N(CH_3)-$, -NHC(O)-, $-CH_2$-S-, -S-CH_2-, -C(O)-, or -O-; and X is additionally a single bond where Y is pyridyl; and

Y is phenyl, pyridyl, naphthyl, pyridone, pyrazine, pyrimidine, benzodioxane, benzopyridine or benzothiazole, each optionally substituted by C_{1-10}alkyl.

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Adenovirus Infection Stimulates the Raf/MAPK Signaling Pathway and Induces Interleukin-8 Expression

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Previous studies have shown that airway administration of adenovirus or adenovirus vectors results in a dose-dependent inflammatory response which limits the duration of transgene expression. We explored the possibility that adenovirus infection triggers signal transduction pathways that induce the synthesis of cytokines and thus contribute to the early inflammatory response. Since stimulation of the Raf/mitogen-activated protein kinase (MAPK) pathway activates transcription factors that control the expression of inflammatory cytokines, we examined the activation of this pathway following adenovirus infection. Adenovirus infection induced the rapid activation of Raf-1 and a transient increase in the tyrosine phosphorylation and activation of $p42^{mapk}$ at early times postinfection. Activation of the Raf/MAPK pathway by adenovirus is likely triggered by the infection process, since it occurred rapidly and with various mutant adenoviruses and adenovirus vectors. Moreover, interleukin-8 (IL-8) mRNA accumulation was evident at 20 min postinfection and was induced even in the presence of cycloheximide. Both MAPK activation and IL-8 production were inhibited by [illegible] a potent inhibitor of Raf-1. These results suggest that adenovirus-induced Raf/MAPK activation contributes to IL-8 production. Adenovirus-induced activation of the Raf/MAPK signaling pathway and IL-8 production may play critical roles in the inflammation observed following in vivo administration of adenovirus vectors for gene therapy.

Recombinant adenoviruses offer many advantages for the development of gene therapy vectors, e.g., the ability to infect nondividing cells, robust transgene expression, and ease of generating high-titer stocks. A major obstacle that stands in the way of effective gene therapy for chronic diseases with adenovirus vectors is transient expression of the therapeutic transgene, which is associated with vector-induced pathology. Two phases of an inflammatory response following adenovirus infection in the lungs of cotton rats and mice have been reported elsewhere (22, 23). The first phase occurs between days 1 and 5 postinfection and is associated with a lymphocyte, monocyte/macrophage, and polymorphonuclear leukocyte infiltration, as well as the local release of the inflammatory cytokines interleukin-1β (IL-1β), tumor necrosis factor alpha (TNF-α), and IL-6. In addition, elevated levels of the inflammatory cytokine IL-6 have been observed in the serum following airway administration of adenovirus vectors in humans (14), rats (22), and mice (23). Recently, increased levels of IL-8 were observed in the bronchoalveolar lavage fluid (BALF) following airway delivery to macaques (67). The second phase occurs 5 to 7 days postinfection and is characterized by perivascular and bronchiolar infiltration of lymphocytes. The T-cell infiltrate, characteristic of the second inflammatory phase, contributes to the transient nature of transgene expression observed following adenoviral gene therapy. $CD4^+$ T cells of the Th-1 subclass produce gamma interferon (IFN-γ), which upregulates expression of major histocompatibility complex class I molecules on vector-transduced cells (74). $CD8^+$ T cells perform the major effector function and destroy vector-transduced cells in an major histocompatibility complex class I-restricted fashion (71–73).

Over the past several years, a clear understanding of a major signaling pathway that controls cell growth has emerged. Stimulation of receptor tyrosine kinases results in receptor clustering and autophosphorylation (63). Guanine nucleotide exchange factors for Ras are then recruited to the cell membrane by adapter proteins which bind to the exchange factors via SH3 domains and interact with the tyrosine-phosphorylated receptors via their SH2 domains (10, 12, 18, 40, 50, 53, 58). The exchange factors then activate Ras by releasing bound GDP and freeing Ras to bind excess GTP in the cytoplasm (55). Ras-GTP recruits Raf-1 to the plasma membrane, where it is subsequently activated by other kinases (19, 34, 39, 60). Activated Raf-1 phosphorylates and activates mitogen-activated protein kinase (MAPK; also known as extracellular signal-regulated kinase [ERK])-activating kinase (MEK) (16, 30, 37), which in turn phosphorylates and activates MAPK. Cytosolic phospholipase A_2 is phosphorylated and activated by MAPK, resulting in the production of arachidonic acid, a potent inflammatory mediator (41). In addition, activated MAPK translocates to the nucleus, where it phosphorylates and activates transcription factors (15).

Activation of the Raf/MAPK pathway has been shown to stimulate transcription through AP-1, ETS, NF–IL-6, and NF-κB elements (8, 9, 20, 65). Transcription of many cytokine genes is regulated by factors that bind to these elements. For example, NF-κB and NF–IL-6 are necessary for IL-1β, TNF-α, IL-6, and IL-8 expression. Moreover, activation of the Raf/MAPK signaling pathway has been shown to trigger the synthesis and release of cytokines. For example, Raf-1 is required for IL-2 production in response to phorbol 12-myristate 13-acetate (TPA) or anti-CD3 antibody, and constitutively activated Raf-1 stimulates IL-2 production in T cells (52).

Cyclic AMP functions as a negative regulator of this pathway by activating protein kinase A (13, 25, 27, 29, 57, 68). Protein kinase A has been reported to inhibit Raf-1 activity by two distinct mechanisms. Cyclic AMP stimulates the phosphorylation of Raf-1 on serine 43, which decreases the affinity of Raf-1 for Ras (68). However, protein kinase A can also inhibit the

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activity of Raf-1 through phosphorylation of its kinase domain (27).

Very little is known concerning the mechanisms that initiate the first phase of inflammation following adenovirus infection. One possibility is that the first phase is elicited by the activation of signaling pathways in the transduced cell that identify it as being infected by a virus. Adenovirus infection may trigger signaling pathways that activate cellular defenses such as cytokine induction and arachidonic acid production.

In this report, we demonstrate that adenovirus infection activates the Raf/MAPK signaling pathway. In addition, our results suggest that activation of this pathway contributes to IL-8 production following adenovirus infection.

MATERIALS AND METHODS

Cells and viruses. HeLa cells were obtained from the American Type Culture Collection and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum. Adenovirus type 5 (Ad5) and deletion mutant dl312 were obtained from the American Type Culture Collection. The E1-, E3-deleted adenovirus AdLacZ expresses the *lacZ* gene from the Ad5 E1 region. AdCMVNull is similar to AdLacZ except that it contains only the cytomegalovirus promoter and simian virus 40 poly(A) sequence in the E1 region. The E1-, E3-deleted adenovirus AdGus expresses β-glucuronidase from the cytomegalovirus promoter in the E1 region.

All viruses were propagated on 293 cells (26). The viruses were purified from infected cells at 2 days postinfection by three freeze-thaw cycles that were followed by three successive bandings on CsCl gradients. Purified virus was dialyzed against a 10 mM Tris (pH 7.8) buffer containing 150 mM NaCl, 10 mM $MgCl_2$, and 3% sucrose and was stored at −70°C until use. All viruses were tested and found to have replication-competent adenovirus levels of less than 1 in 10^7 PFU. Heat-inactivated virus was generated by incubating the virus preparation at 56°C for 1 h.

Immunoblotting and kinase assays. Cells grown on 10-cm plates were starved in serum-free DMEM for 18 h and then treated with 100 ng of TPA per ml (Gibco-BRL) or were infected with adenovirus vectors at a multiplicity of infection (MOI) of 50 in 1 ml of serum-free DMEM, unless otherwise indicated. At the indicated time points, the cells were washed three times with phosphate-buffered saline (PBS) and lysed in 1 ml of ice-cold RIPA buffer (20 mM Tris [pH 7.4], 137 mM NaCl, 10% glycerol, 0.1% sodium dodecyl sulfate [SDS], 0.5% deoxycholate, 1% Triton X-100, 2 mM EDTA containing 25 mM glycerol phosphate, 10 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 5 μg of aprotinin per ml, 5 μg of leupeptin per ml, and 1 mM sodium vanadate) at 4°C for 10 min. Cell lysates were scraped into Eppendorf tubes and clarified. The supernatant was then transferred to a new tube and used for immunoprecipitation with either a Raf-1-specific antiserum generated against the C-terminal domain of Raf-1 or ERK-2-specific antiserum (Santa Cruz Biotechnologies). Immunoprecipitates were washed three times with RIPA buffer for immunoblotting and twice each with RIPA buffer, LiCl buffer (0.5 M LiCl, 0.1 M Tris [pH 8]), and kinase buffer (25 mM HEPES [pH 7.4], 25 mM glycerol phosphate, 1 mM dithiothreitol [DTT], 10 mM $MgCl_2$) for kinase assays. MAPK assays were performed in 40-μl reaction mixtures with 10 μCi of [γ-^{32}P]ATP–10 μM unlabeled ATP–2 μg of myelin basic protein (MBP). Raf kinase assays were performed in 40-μl reaction mixtures with 10 μCi of [γ-^{32}P]ATP–10 μM unlabeled ATP–0.5 μg of MKK K97M (42). Kinase reaction mixtures were incubated for 30 min at room temperature. Laemmli buffer was added, and the reaction mixtures were boiled for 5 min and electrophoresed on SDS-polyacrylamide gels. The gels were either dried down and exposed or transferred to nitrocellulose, exposed, and subsequently used for immunoblots to control for equal levels of Raf-1 or MAPK in the immunoprecipitates.

For immunoblot analysis, immunoprecipitates were electrophoresed on SDS-polyacrylamide gels, electroblotted to nitrocellulose, blocked with TBST (10 mM Tris [pH 8], 150 mM NaCl, 0.05% Tween-20) containing 2% bovine serum albumin for 30 min at room temperature, and incubated with either ERK-2, Raf-1, or phosphotyrosine specific-antiserum at room temperature for 1 h. The immunoblots were washed in TBST and incubated with either antimouse or anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Boehringer Mannheim) for 50 min at room temperature and then washed in TBST and detected by enhanced chemiluminescence (Amersham).

Protein purification. pRSET A +MKK (K97M) (a gift from N. Ahn) was used to transform the BL21 strain of *Escherichia coli*. A culture was incubated at 37°C until the optical density at 590 nm reached 0.5. The temperature was shifted to 25°C, the culture was induced with 0.05 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and the cells were grown for an additional 20 h (1). The bacterial culture was pelleted and lysed in lysis buffer containing 20 mM triethanolamine, 10 mM Tris (pH 7.8), 60 mM NaCl, 0.05% (wt/vol) Triton X-100, 2 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin per ml, 2 μM pepstatin, and 10 μg of aprotinin per ml. Lysozyme was added to 1 mg/ml, and the lysate was incubated on ice for 30 min and then sonicated. DNase I (Boehringer Mannheim, grade II) was added to a final concentration of 25 μg/ml, $MgCl_2$ was added to a final concentration of 2.5 mM, and the mixture was incubated on ice for 10 min. The material was centrifuged at 100,000 × *g* for 30 min at 4°C. Ni^{2+} nitrilotriacetic acid (NTA)-agarose purification was performed as previously described (31). Briefly, the supernatant was adjusted to 17% glycerol and incubated with 4.5 ml of washed Ni^{2+} · NTA-agarose resin (Qiagen) for 1 h with constant agitation. The material was centrifuged at 200 × *g* for 10 min at 4°C and washed with and resuspended in buffer D (10 mM HEPES [pH 7.9], 5 mM $MgCl_2$, 0.1 mM EDTA, 50 mM NaCl, 1 mM DTT, 17% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, 10 mM NaF) containing 0.8 mM imidazole. The resin was washed successively with buffer D containing 0.8 mM and then 8 mM imidazole and was then eluted with buffer D containing 40 mM and then 80 mM imidazole. The fractions containing MKK (K97M) were pooled and dialyzed for 4 h in buffer containing 25 mM Tris (pH 7.4), 5 mM EGTA, 2 mM DTT, 0.1% (wt/vol) Triton X-100, and 50% (vol/vol) glycerol and were stored at −20°C.

Northern (RNA) analysis. HeLa cells were infected with AdCMVNull at a MOI of 50 or treated with TNF-α per ml (Boehringer). Following infection, cells were washed three times with PBS, and total RNA was extracted by using RNAzol B (Biotecx Laboratories, Inc., Houston, Tex.) following the manufacturer's instructions. A 10-μg amount of total RNA was electrophoresed on a 1% agarose formaldehyde gel at 100 V for 2 h. RNA was transferred to a nylon membrane and cross-linked with 120,000 μJ with a Stratalinker UV cross-linker (Stratagene). Northern blots were hybridized with ^{32}P-labeled IL-8 cDNA and GAEDH cDNA fragments. The blots were washed and exposed to film.

IL-8 ELISA. HeLa cells were plated at a density of 2 × 10^5 cells per well in 12-well plates. After 24 h, the cells were incubated with forskolin or 2′,5′-dideoxyforskolin at a concentration of 20 μg/ml in 0.25 ml of DMEM supplemented with 2% calf serum for 10 min at 37°C. AdLacZ at a MOI of 100 was added, and the mixture was incubated for 60 min at 37°C. The infected cells were washed with PBS and then incubated in 1 ml of DMEM supplemented with 2% calf serum at 37°C in a humidified incubator at 5% CO_2. After 19 h, the cell medium was analyzed for IL-8 protein by a sandwich enzyme-linked immunosorbent assay (ELISA) (R&D Systems) according to the manufacturer's instructions.

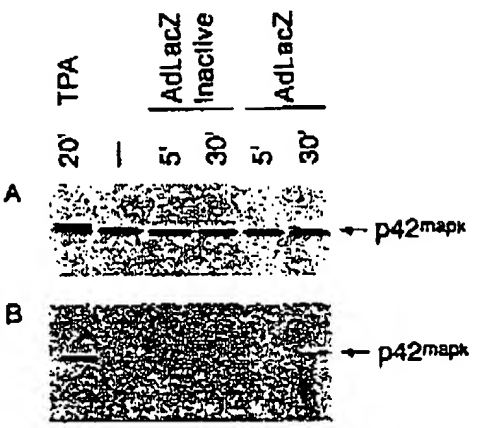

FIG. 1. Adenovirus infection induces the tyrosine phosphorylation and mobility shift of MAPK. HeLa cells were treated with TPA for 20 min or were infected with AdLacZ or heat-inactivated AdLacZ for 5 and 30 min. (A) Cell lysates were immunoprecipitated and then immunoblotted with an $p42^{mapk}$-specific antiserum. (B) Tyrosine phosphorylation of $p42^{mapk}$ was analyzed by immunoblotting $p42^{mapk}$ immunoprecipitates with the 4G10 anti-phosphotyrosine antibody.

RESULTS

Adenovirus infection activates MAPK. To determine if adenovirus infection stimulates the Raf/MAPK signal transduction pathway, HeLa cells were infected with a highly purified adenovirus vector, AdLacZ. Cell lysates were obtained 5 and 30 min after the initiation of infection and were immunoprecipitated with $p42^{mapk}$-specific antiserum. Immunoblotting of the $p42^{mapk}$ immunoprecipitates with $p42^{mapk}$ antiserum revealed the existence of a shifted form of $p42^{mapk}$ present 30 min after infection with the vector or stimulation with TPA (Fig. 1A). Virus that was heat inactivated prior to infection

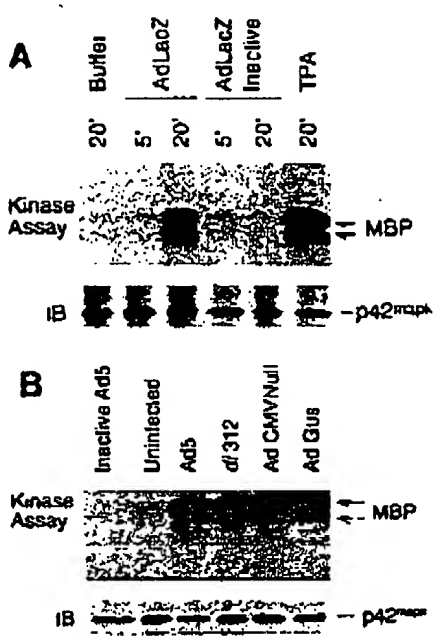

FIG. 2. Adenovirus infection activates MAPK. Cell lysates were analyzed for MAPK activity with $p42^{mapk}$ immunoprecipitates in immune complex kinase assays with MBP as the substrate. The bottom panels show $p42^{mapk}$ immunoblots (IB) of the material used in the kinase assays. (A) HeLa cells were mock infected (buffer) or were infected with AdLacZ or heat-inactivated AdLacZ at a MOI of 25 PFU per cell for 5 and 20 min or were stimulated with TPA as indicated. (B) HeLa cells were infected with Ad5, *dl*312, AdCMVNull, AdGus, or heat-inactivated Ad5 at a MOI of 6,000 particles per cell for 20 min.

failed to induce the $p42^{mapk}$ mobility shift. Previous studies have correlated the altered mobility of $p42^{mapk}$ with the tyrosine phosphorylation and activation of $p42^{mapk}$. To determine if the shifted form of $p42^{mapk}$ was phosphorylated on tyrosine, a similar immunoblot was probed with the 4G10 antibody. Tyrosine-phosphorylated $p42^{mapk}$ similar to that observed following TPA treatment was detected in lysates from cells infected with the adenovirus vector, but not the heat-inactivated vector (Fig. 1B).

To determine if this modified form of $p42^{mapk}$ had increased kinase activity, cell lysates from vector-transduced cells were immunoprecipitated with $p42^{mapk}$-specific antiserum, and in vitro kinase assays were performed with myelin basic protein as a substrate. A dramatic increase in $p42^{mapk}$ kinase activity was evident at 20 min postinfection that was not seen with the heat-inactivated vector (Fig. 2A, top panel). The elevation in $p42^{mapk}$ kinase activity was similar in magnitude to that observed following TPA treatment of the cells. Immunoblot analysis of the filter with $p42^{mapk}$-specific antiserum revealed that equivalent levels of MAPK protein were present in the immunoprecipitates (Fig. 2A, bottom panel). These results indicate that the tyrosine phosphorylation and specific activity of MAPK are increased dramatically following adenovirus infection. To determine if the elevation in MAP kinase activity was specific to AdLacZ, cells were infected with wild-type Ad5, an E1a deletion mutant, *dl*312, or two adenovirus vectors, AdCMVNull or AdGus. In vitro MAP kinase assays were performed on $p42^{mapk}$ immunoprecipitates from cell lysates harvested 20 min after infection. All four viruses, but not heat-inactivated Ad5, activated $p42^{mapk}$ efficiently (Fig. 2B). These results indicate that activation of MAPK is not specific for AdLacZ but appears to be a general effect of adenovirus infection.

Different extracellular stimuli can result in either transient or sustained activation of $p42^{mapk}$ in PC12 cells (28, 49, 61). Transient activation of MAPK results in proliferation, whereas differentiation is the response to sustained activation. A time course of $p42^{mapk}$ kinase activity following adenovirus infection revealed detectable $p42^{mapk}$ activation 2 min after infection (Fig. 3, top panel). $p42^{mapk}$ kinase activity peaked at 20 min after infection and subsequently waned, with only minor elevation observed after 60 min. Immunoblot analysis with $p42^{mapk}$-specific antiserum revealed that equivalent levels of MAPK protein were present in the immunoprecipitates (Fig. 3, bottom panel). We conclude that adenovirus infection results in a transient activation of $p42^{mapk}$.

Adenovirus infection activates Raf-1. Activation of MAPK has been reported to occur via two distinct pathways, one stimulated by Raf-1 (16, 30, 37, 62) and the second stimulated by MEK kinase (38). The physiological significance of the MEK kinase induced activation of MAPK has recently been questioned, since MEK kinase has been shown to be a potent stimulator of the stress-activated protein kinases and appears to stimulate MAPK only when overexpressed (17, 69, 70). To determine if adenovirus infection-induced MAPK activation is mediated by Raf-1, in vitro kinase assays were performed with Raf-1 immunoprecipitates and kinase-inactivated MEK as substrate. This analysis demonstrated that the AdLacZ vector, but not the inactivated vector, increased the phosphotransferase activity of Raf-1 (Fig. 4).

A time course of Raf-1 activation following adenovirus infection revealed that Raf-1, like MAPK, was maximally activated at 20 min postinfection (data not shown). This 20-min delay in the activation of Raf may be due to a delay in adenovirus binding to its receptor. To address this issue and determine the timing of Raf kinase activation following receptor binding, temperature shift experiments were performed. Adenovirus will bind to but will not enter cells efficiently at 4°C (26, 64). Adenovirus was added to cells at 4°C and was allowed 60 min to bind to cells. The cells were then warmed to 37°C, and Raf kinase activity was monitored as a function of time. Under these conditions, maximal Raf-1 activation was observed 5 min after the shift from 4 to 37°C (Fig. 5). These results suggest

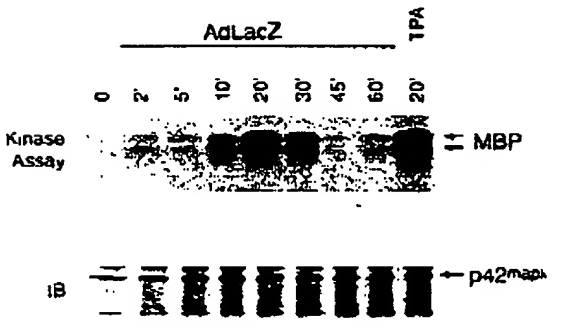

FIG. 3. Time course of MAPK activation following adenovirus infection. HeLa cells were infected with AdLacZ for various times or were stimulated with TPA as indicated. Cell lysates were analyzed for MAPK activity with $p42^{mapk}$ immunoprecipitates in immune complex kinase assays with MBP as the substrate. The bottom panel shows a $p42^{mapk}$ immunoblot (IB) of the material used in the kinase assay.

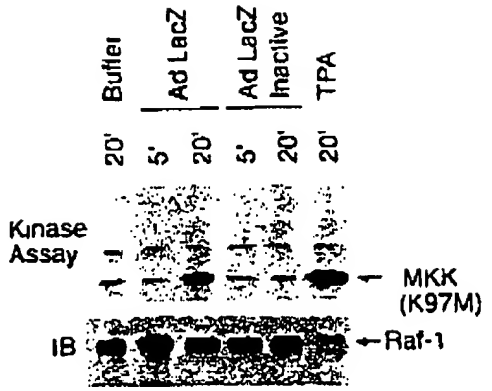

FIG. 4. Adenovirus infection activates Raf-1 kinase. HeLa cells were treated with TPA and infected with AdLacZ or heat-inactivated AdLacZ for the indicated periods of time. Cell lysates were immunoprecipitated with a Raf-1-specific antiserum, washed, and then incubated with inactivated MAPK kinase (MKK K97M) in the presence of [γ-^{32}P]ATP. Phosphorylated MKK K97M was resolved on a 10% polyacrylamide-SDS gel, transferred to nitrocellulose, and exposed. The bottom panel shows a Raf-1 immunoblot (IB) [illegible] kinase assay.

that Raf-1 is activated early after adenovirus binds to its receptors.

Adenovirus infection induces IL-8 synthesis. We then investigated the possibility that adenovirus-induced activation of this pathway results in the induction of cytokine synthesis. Since IL-8 is produced by epithelial cells (2, 35, 46, 59) in response to various stimuli, IL-8 mRNA levels were examined at early times following adenovirus infection. HeLa cells were infected with AdCMVNull, an adenovirus vector similar to AdLacZ, except that it does not express a transgene, and IL-8 mRNA levels were determined at various times postinfection by Northern analysis. Detectable levels of IL-8 mRNA were observed 20 min after infection, peaked at 60 min, and were

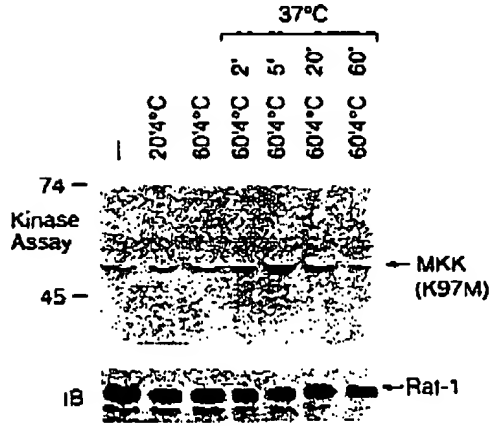

FIG. 5. Rapid activation of Raf-1 after adenovirus infection. HeLa cells were incubated with AdLacZ for 20 or 60 min at 4°C and then incubated at 37°C as indicated. Cell lysates were immunoprecipitated with a Raf-1-specific antiserum, washed, and then incubated with inactivated MAPK kinase (MKK K97M) in the presence of [γ-^{32}P]ATP. Phosphorylated MKK K97M was resolved on a 10% polyacrylamide-SDS gel, transferred to nitrocellulose, and exposed. The bottom panel shows a Raf-1 immunoblot (IB) of the material used in the kinase assay.

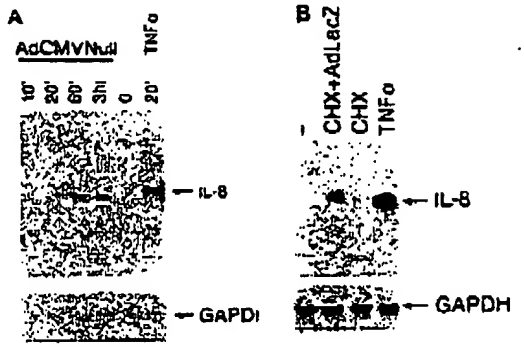

FIG. 6. Adenovirus infection induces IL-8 synthesis. A 10-μg amount of total cellular HeLa cell RNA was analyzed with IL-8 and GAPDH probes. (A) RNA was harvested at the indicated times following infection with AdCMVNull at a MOI of 25 or after stimulation with 10 ng of TNF-α per ml. (B) RNA was harvested at 60 min after infection with AdLacZ in the presence or absence of 5 μg of cycloheximide per ml as indicated.

sustained for up to 3 h (Fig. 6A). Similar results were obtained with the AdLacZ vector (Fig. 6B). IL-8 expression was not detectable in uninfected control cells. The kinetics of IL-8 induction following adenovirus infection suggest that it is triggered by a posttranslational event. Infections performed in the presence of cycloheximide support this idea, since cycloheximide had no effect on adenovirus-induced IL-8 induction (Fig. 6B). The migration of IL-8 mRNA induced by TNF-α is slightly slower than that induced by adenovirus infection. The reason for this is not clear but may be due to altered processing of the IL-8 message or differential transcription start site utilization. Importantly, the increase in IL-8 mRNA levels following adenovirus infection correlated with an increase in secreted IL-8 protein observed in the media by an ELISA (Fig. 7B).

Activation of the Raf/MAPK pathway is required for adenovirus-induced IL-8 induction. To determine if Raf-1 is necessary for adenovirus induction of IL-8, the Raf/MAPK signaling pathway was blocked with forskolin, a drug that increases cyclic AMP levels and activates protein kinase A (56). Immunoprecipitates of $p42^{mapk}$ from cells infected with adenovirus in the presence of forskolin were severely compromised in their ability to phosphorylate MBP, while 1,9-dideoxyforskolin (an inactive forskolin analog) had no effect (Fig. 7A). We then looked at the effect of blocking the Raf/MAPK signaling pathway on IL-8 synthesis. Cells were infected with adenovirus vector AdLacZ, in the presence or absence of forskolin or 1,9-dideoxyforskolin and IL-8 protein levels in the media were analyzed 19 h postinfection by ELISA. Pretreatment of cells with forskolin, but not 1,9-dideoxyforskolin, attenuated vector-induced IL-8 production (Fig. 7B). These results indicate that forskolin blocks adenovirus infection-induced activation of the Raf/MAPK pathway and suggest that the Raf/MAPK signaling pathway is necessary for maximal IL-8 synthesis following infection.

DISCUSSION

To gain a better understanding of the inflammatory response following adenovirus infection, we examined cellular signal transduction pathways that are activated following adenovirus infection. Our results demonstrate that both Raf-1 and MAPK are activated at early times following adenovirus infection. The

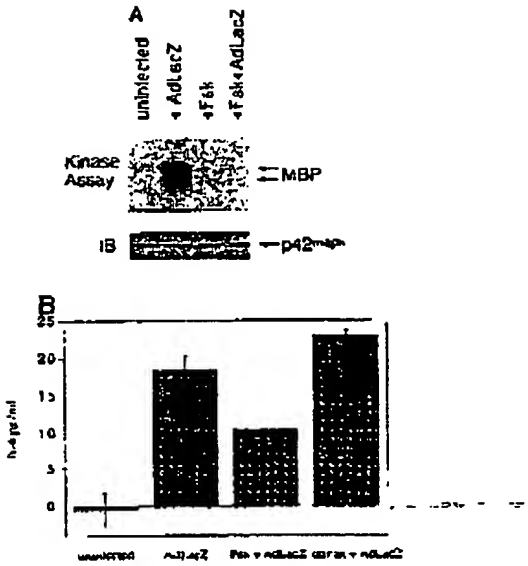

FIG. 7. Forskolin inhibits MAPK activation and IL-8 synthesis following AdLacZ infection (A) HeLa cells were left untreated or treated with forskolin (Fsk) for 20 min. Cells were then infected with AdLacZ for 20 min, and cell lysates were analyzed for MAPK activity by using $p42^{mapk}$ immunoprecipitates in immune complex kinase assays with MBP as substrate. The bottom panel shows an $p42^{mapk}$ immunoblot (IB) of the material used in the kinase assay. (B) HeLa cells were incubated with forskolin (Fsk) or 1,9-dideoxyforskolin (ddFsk) at a concentration of 20 μg/ml for 10 min at 37°C. The cells were then infected with AdLacZ at a MOI of 100 for 60 minutes at 37°C. After 19 h, the cell medium was analyzed for IL-8 protein by a sandwich ELISA. Error bars indicate the standard errors of the means ($n = 3$).

activation of this signaling pathway may be important in the initiation of immune and inflammatory responses against the virus.

Infiltration of leukocytes during an inflammatory response is mediated by chemotactic factors such as IL-8. IL-8 is a potent chemotactic cytokine for neutrophils and T lymphocytes (51). Induced expression from the IL-8 promoter is dependent on three distinct enhancer elements, the AP-1, NF-κB, and NF–IL-6 binding sites. TNF-α and gamma interferon synergistically activate the IL-8 promoter through the NF-κB and AP-1 elements in a human gastric cancer cell line (75). The NF-κB and the NF–IL-6 binding sites are essential for activation of the IL-8 promoter in a human T-cell line, Jurkat, and in a human monocytic cell line, U937 (36, 44). Each of these elements has previously been shown to be regulated by the Raf/MAPK signaling pathway. MAPK phosphorylates NF–IL-6 at Thr-235, resulting in an increase in NF–IL-6 transcriptional activity (45). Transcriptional transactivation through AP-1 sites (8, 65) and NF-κB binding sites (9, 20) is stimulated by Raf-1.

Adenovirus-induced IL-8 expression has recently been shown in A549 cells (2). These investigators demonstrated an increase in IL-8 mRNA and secreted protein 24 h after infection. Thus, a paracrine mechanism for IL-8 production following infection could not be ruled out. Our results indicate that IL-8 mRNA accumulates as early as 20 min after cells are exposed to the virus. Since cycloheximide did not block the rapid accumulation of IL-8 mRNA, it appears that an adenovirus-induced activation of the IL-8 promoter occurs by a post-translational mechanism. It is likely that adenovirus-induced activation of the Raf/MAPK pathway contributes to the induction of the IL-8 promoter since forskolin, a potent inhibitor of Raf-1, inhibited both MAPK activation and IL-8 induction following adenovirus infection, while the inactive analog 1,9-dideoxyforskolin had no effect.

The mechanism by which adenovirus infection triggers the Raf/MAPK signaling pathway is not known. It is unlikely that expression of any gene products from the adenovirus vectors used in this study are involved in activating the pathway, since it is activated by many different vectors that express different genes. Moreover, the timing of Raf-1 and MAPK activation is not consistent with gene expression playing a role, since these kinases are activated between 2 and 20 min postinfection, a time when no virus-specific gene expression is expected to occur. Adenovirus binding to cells is dependent on an interaction of fiber with an unknown cell surface receptor. The virus particles are then rapidly internalized by receptor-mediated endocytosis. Viral entry is accelerated by the interaction of penton base with αv integrins (3, 66). Since ligation of integrins has been shown to stimulate the MAPK pathway (11, 33, 43, 54), it is possible that the penton base integrin interaction mediates activation of the Raf/MAPK pathway. Alternatively, fiber binding to its receptor or subsequent events, such as endosome rupture, may play a role. These possible mechanisms of Raf/MAPK pathway stimulation by adenovirus infection are currently being examined.

The finding that adenovirus infection activates the Raf/MAPK signaling pathway raises the possibility that activation of this pathway is necessary for efficient adenovirus infection. There are several steps in the infection process that may be regulated by the activation of the Raf/MAPK signaling pathway. Adenovirus E1A proteins have many functions, one of which is to bind to Rb and p107 and thereby activate E2F. This function of E1A is thought to induce quiescent cells to enter S phase, an environment conducive for viral DNA synthesis (47). Since vRaf is an oncogene and many mitogenic agents activate the Raf/MAPK pathway, it is conceivable that adenovirus-induced activation of the Raf/MAPK pathway may facilitate the cell cycle progression of quiescent cells, thus setting the stage for efficient viral replication. A second possible benefit for the virus suggested by the activation of the Raf/MAPK pathway following adenovirus infection may be to drive E1A transcription more efficiently. E1A is the first gene to be transcribed during an adenovirus infection (48), and its transcription is dependent on cellular transcription factors. Expression of the remaining adenovirus early genes requires E1A (4, 32). Thus, the initiation of E1A transcription is a critical step in the infection process. E1A transcription is controlled by an Ets family transcription factor, EF-1A, which is the human homolog of GABP (5–7). We have recently shown that activation of the Raf-1 signaling pathway results in the activation of GABP (21). Thus, it is likely that adenovirus infection-induced activation of the Raf/MAPK pathway augments E1A transcription and helps to initiate the early phase in adenovirus infection. Other steps in the viral life cycle could be affected by the activation of the Raf/MAPK pathway, such as increased efficiency in viral uncoating, transport, or enhanced translation of viral transcripts. Alternatively, it is possible that activation of this pathway is not important for efficient infection and that it is a side effect of infection. These possibilities are currently being examined.

The implications of this work for adenovirus-based gene therapy are that vector-induced activation of the Raf/MAPK signaling pathway and IL-8 production may contribute to the host inflammatory response observed following adenovirus vector delivery. Blocking these pathways may be critical for

developing an adenovirus delivery system that is associated with reduced inflammation.

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